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# Characterizing the Role of *Phaeobacter* in the Mortality of the Squid, *Euprymna scolopes*

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Characterizing the Role of *Phaeobacter* in the Mortality of the Squid,  
*Euprymna scolopes*.

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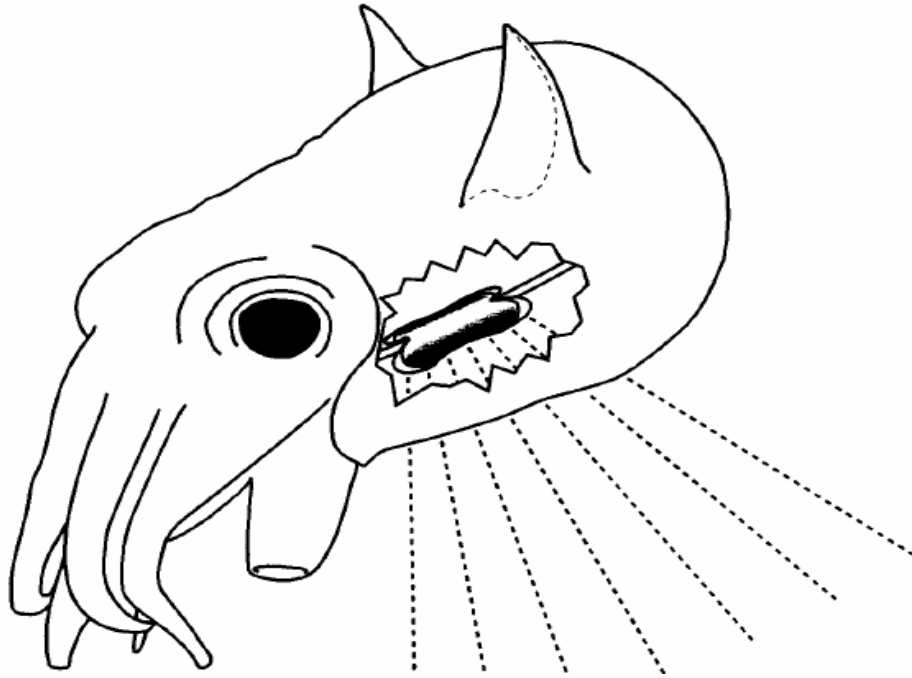
## Abstract

The subject of our study is the Hawaiian bobtail squid, *Euprymna scolopes*, which is known for its model symbiotic relationship with the bioluminescent bacterium, *Vibrio fischeri*. The interactions between *E. scolopes* and *V. fischeri* provide an exemplary model of the biochemical and molecular dynamics of symbiosis since both members can be cultivated separately and *V. fischeri* can be genetically modified<sup>1</sup>. However, in a laboratory setting, the mortality of embryonic *E. scolopes* can be a recurrent problem. In many of these fatalities, the egg cases display a pink-hued biofilm, and rosy pigmentation has also been noted in the deaths of several adult squid. To identify the microbial components of this biofilm, we cloned and sequenced the 16s ribosomal DNA gene from pink, culture-grown isolates from infected egg cases and adult tissues. One of the culture-grown species was identified as *Phaeobacter*, a genus of bacteria related to *Roseobacter*, a resident component of the accessory nidamental gland (ANG), an organ involved with the secretion of the jelly egg coat in squids. To establish if *Phaeobacter* was acting as a pathogen, we infected juvenile *E. scolopes* with concentrations of *Phaeobacter* ranging from  $10^3$  to  $10^6$  cells per milliliter of filter-sterilized sea water. Our data suggests that *Phaeobacter* may be a causative agent in the deaths of juvenile squids. Current molecular analyses seek to identify *Phaeobacter* as a resident of ANG tissue, as well as identify and obtain cultured isolates of other bacteria that may reside in the ANG. Symbiotic relationships can potentially turn pathogenic. For example, resident and normally benign bacteria of the human gastrointestinal tract, *Helicobacter pylori* and *Escherichia coli*, contribute to disease in a small segment of the human population. Hence, understanding the mechanisms whereby resident microbiota become pathogenic in the model squid *E. scolopes*, may have broader implications in understanding the progression of some infectious diseases.

## **Introduction**

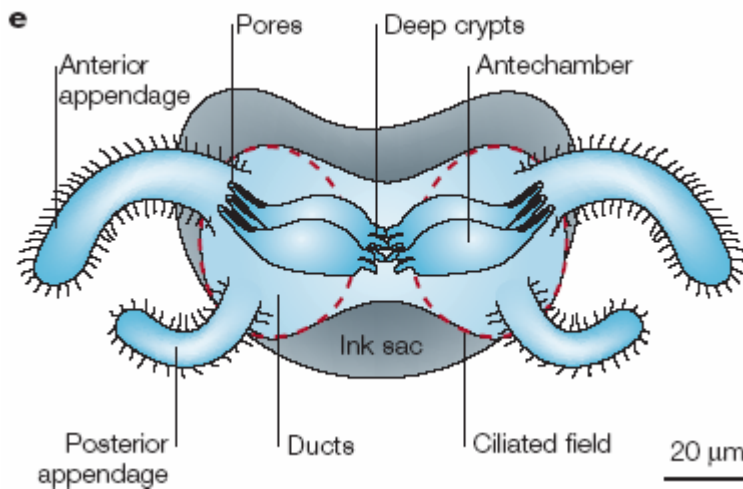
In nature, microbes interact with animal hosts to produce viable arrangements. While most of these associations are benign and sometimes even beneficial, others produce adverse effects on the health of the host organism. *Escherichia coli* is a microbial resident of the human gastrointestinal tract that naturally produces menaquinone or vitamin K<sub>2</sub>, which is essential in the maintenance of healthy bones; however, in some cases, *E. coli* infection gives rise to hemorrhagic colitis, substantiating that there is a fine line between pathogenesis and symbiosis. Genetic and molecular analyses have identified common cell signaling mechanisms involved in the colonization of both pathogenic and symbiotic bacteria. Understanding these interactions between a bacterium and its host may provide valuable insight into the development of disease and the establishment of healthy microbiota.

*Euprymna scolopes* is a species of sepiolid squid endemic to Hawaiian shores. A primarily nocturnal creature, *E. scolopes* buries itself in the sand of the ocean floor throughout the day. At night, it deters potential predators by a process known as counterillumination, in which the squid emits light from its ventral surface to camouflage itself in down-bearing lunar light (Fig. 1). This survival advantage is made possible by a Gram negative, luminous bacterium, *Vibrio fischeri*, which colonizes the light organ of the Hawaiian bobtail squid. As in many other microbial associations, the symbiont colonizes from the environment by horizontal transmission.



**Figure 1.** This illustration of *E. scolopes* displays light emitted from the ventral surface of the squid. Depicted by the cylindrical structure, the light organ is the source of the squid's luminescence<sup>2</sup>.

The light organ of *E. scolopes* is located in the medio-central portion of the mantle cavity, and hosts a monospecific culture of *V. fischeri*. Prior to colonization by the bioluminescent symbiont, it remains free of nonsymbiotic bacteria. The light organ is divided into two lobes, each endowed with three crypt spaces, lined with epithelial cells<sup>1</sup>. The crypt spaces are connected to pores on the latero-ventral surface of the light organ by ciliated ducts. Each set of pores are a part of a ring-shaped base that features two protruding appendages (Fig. 2).



**Figure 2.**

An internal view of the squid light organ at the time of hatching<sup>1</sup>.

In Hawaiian seawater, *Vibrio fischeri* is a relatively scarce member of the natural microbiota. Outside of dense coastal populations of the Hawaiian bobtail squid, *V. fischeri* comprise less than 0.1% of the bacterioplankton population<sup>1</sup>. Thus, the colonization of the nascent light organ by *V. fischeri* must be mediated by the physiological and biochemical responses of the developing host. While dormant in the daytime, *E. scolopes* typically shed up to 95% of light organ symbionts into the surrounding water; the remaining *V. fischeri* recolonize the light organ. This action ensures that mature *E. scolopes* have a new, complete set of symbionts for nocturnal activities, and as a result, establishes an environment conducive for *V. fischeri* to interact with newly hatched, aposymbiotic juvenile squid<sup>1</sup>.

The *E. scolopes*-*V. fischeri* relationship serves as a model for the study of symbiosis for several reasons. First, the association that occurs in the squid light organ is specific to *V. fischeri*; in a lab, infection by *V. fischeri* is quickly and effectively verified by the measurements of a luminometer, in which symbiotic squids typically return

relative light units (RLUs) of 1000 and up. In addition, since *E. scolopes* hatch aposymbiotic, the initiation of the infection by the symbiont can be experimentally controlled. This is can be done with relative ease because both host and bacterium can be cultured independently. With the publication of the *V. fischeri* genome, molecular genetics can be applied to produce genetic variants. A recent study examining the host immune response utilized the OM3 mutant, in which the gene encoding the outer membrane protein was knocked out<sup>3</sup>. Finally, the *E. scolopes*-*V. fischeri* model represents a microbial association prevalent in nature – the colonization of host epithelia by gram negative extracellular bacteria.

### **The Accessory Nidamental Gland**

The female reproductive systems of several taxa of cephalopods (including the bobtail squid family, Sepiolidae) bear a common organ known as the accessory nidamental gland (ANG). The distinct function of the ANG is unknown but it is suspected to play a role in the secretion of the gelatinous outer coat in the squid eggs. In several squid species, the ANG was found to harbor a microbial consortium. Like the light organ, the bacterial species residing in the ANG are horizontally transmitted. At the point of sexual maturity, the carotenoids produced by the resident microbiota change the color of the ANG from a creamy white to a reddish orange. One study on the reef squid, *Loligo opalescens*, reported non-luminescent bacteria associating with the outer sheath of squid egg capsules<sup>4</sup>; they reported that the eggs of *Loligo opalescens* seemed to “suffer little animal, fungal, or microbial attrition<sup>4</sup>.”

The resiliency of these squid eggs was attributed to the possible ability of sheath bacteria to out-compete predatory microbes, or to actively produce antimicrobial substances. As of now, these hypotheses remain mostly speculation. Describing the bacteria present in the ANG of the Hawaiian bobtail squid may provide insight in the survival mechanism of the nascent juvenile eggs.

### **The Problem**

In the laboratory, adult *E. scolopes* are paired by sex in tanks to promote mating. During their nocturnal activity, they are liable to lay their eggs on several surfaces including the walls of the aquarium or portions of PVC pipe. In the daytime, egg clutches are removed to be housed in individual beakers of aerated seawater, which facilitates the harvesting of juvenile squid for experimental applications.

During the development of squid embryos into hatchlings, a highly pigmented rouge biofilm has occasionally been noted on the egg clutches. The presence of this biofilm is correlated with the mortality of juvenile squid prior to hatching. Hatchlings produced from clutches with a characteristic pink hue have never been observed. Furthermore, several adult deceased adult squid were recovered with unusually pink tissues. Microscopy with DAPI staining methods confirmed the presence of bacteria on the pink eggs. Taken together, these observations led to the investigation of a potential pathogen as a cause for this recurrent problem. The first step was to characterize and identify the biofilm by extracting DNA from affected egg clutches. The next task was to identify the origin of this biofilm. Published information about the ANG in analogous squid species directed all inquiries to the gland's native microflora as a source. To find a



coincident microbe, it was necessary to characterize all bacterial inhabitants of the ANG in *E. scolopes*.

## **Materials and Methods**

### **Specimen collection**

A pink-colored egg was taken from an affected clutch and streaked on a saltwater tryptone plate (per liter: 5g tryptone, 3g yeast extract 3 ml glycerol, 700 ml filtered seawater, 300 ml dH<sub>2</sub>O, 15 g agar). These plates were grown at 28°C for 24 hours resulting in beige colonies that turned pink after additional 48-72 hour incubation at room temperature. A single colony was used to grow an overnight culture in liquid SWT. The Qiagen Dneasy Blood & Tissue kit was used for the ensuing DNA extraction. By the same method, DNA was extracted directly from ANG tissue.

### **Bacterial characterization**

#### *Gram staining*

Pink Egg bacteria was subjected to standard Gram staining methods.

#### *Motility*

Pink Egg DNA was inoculated in motility agar containing 0.4% in SWT. A positive test was confirmed by the appearance of bacterial growth from the point of inoculation.

#### *Oxidase Test*

Pink Egg DNA was subjected to oxidase testing on Millipore paper. A positive test was noted by a change of color, from violet to purple.

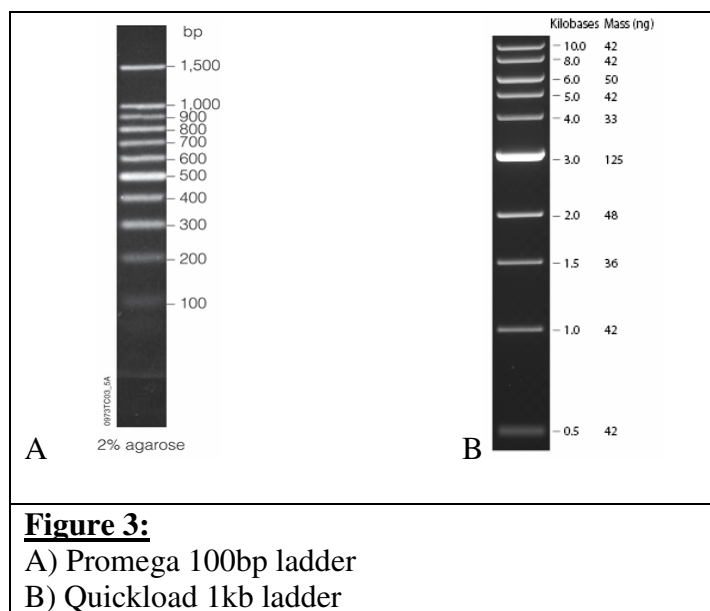
## Amplification 16s rDNA

### *Polymerase Chain Reaction*

Amplification of 16s gene was conducted via a Polymerase Chain reaction (PCR). The cycling conditions included a 95°C denaturation step, a 55°C annealing step, and a 68°C extension step. For a 50 µl reaction, the reagents are listed as follows: 10 µl of 1x Crimson *Taq* reaction buffer (New England BioLabs), which incorporates Tricine, KCl, MgCl<sub>2</sub>, Dextran, and acid red; 0.25 µl of Crimson *Taq* DNA polymerase; 1 µl of a 10 mM Deoxyribonucleotide triphosphate (dNTPs) sample; Universal primers, which target the highly conserved prokaryotic 16s rDNA sequence – 1 µl of a 10 µM sample of 1406R primer (5'-ACGGGCGGTGTGTRC-3'); 1 µl of a 10 µM sample of the 27F primer (5'-AGAGTTTGATCMTGGCTCAG-3'); Template DNA was added to a final concentration of 50-100 ng/ µl. For quality control purposes two DNA samples were used for both the egg biofilm (Pink Egg 1&2), and the accessory nidamental gland (ANG 1&2). ). All reactions were brought to a final volume of 50ul with microbiology grade H<sub>2</sub>O.

### *Gel electrophoresis*

Products generated by PCR were checked for appropriate size using a 1% agarose gel made with 1X TAE (Tris buffer, Acetate, EDTA). The gel was stained by SYBR safe DNA stain (Invitrogen). The expected size of the amplified DNA was ~ 1379 bp in length. The two molecular weight markers were used interchangeably. They were a 100 bp ladder (Promega) and the Quickload 1kb ladder (New England BioLabs).



### *Cleanup*

A PCR cleanup was conducted by the Wizard<sup>®</sup> SV Gel and PCR Clean-Up System (Promega).

### Preparation for Transformation

#### *Preparing for ligation into a plasmid*

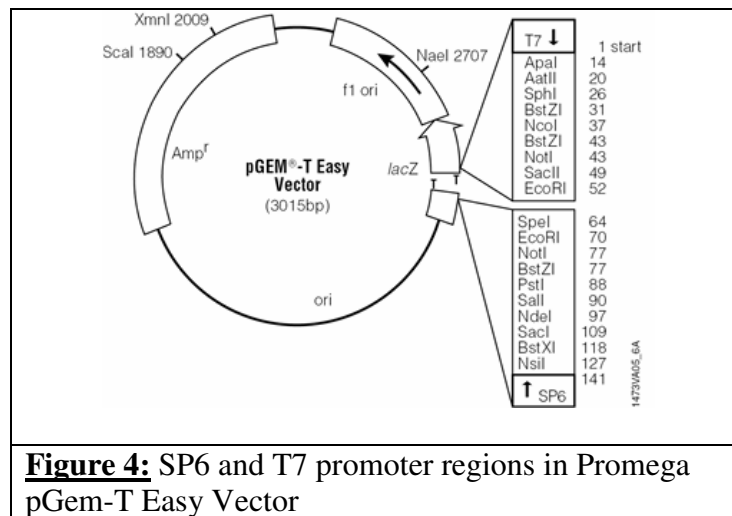
The purified DNA sample was polyadenylated using the following reagents (a final volume of 10 µl): 7 µl of the PCR product; 2 µl of Crimson *Taq* reaction buffer; 0.2 µl of a 10mM sample of dATP (Sigma); 1 µl of Crimson *Taq* DNA polymerase; The thermocycling conditions are as follows:

- 1) 95°C 3 minutes
- 2) 70°C 30 minutes

3) hold at 8°C

### *Overnight ligation*

The polyadenylated DNA was ligated to the poly-T overhangs on the plasmid, disrupting the *lacZ* gene of the linear pGem<sup>®</sup> T-Easy vector to make a circular plasmid. The vector had ampicillin resistance and the SP6 & T7 promoter regions. The ligation reagents for a 10 µl reaction were as such: 5 µl of 2x ligation buffer(Promega); 1 µl of T-Easy vector; 3 µl of PCR product; 1 µl of DNA ligase (Promega); the ligation reaction occurred overnight at 4°C.



**Figure 4:** SP6 and T7 promoter regions in Promega pGem-T Easy Vector

### Transformation

Transformation of *Escherichia coli* cells was conducted by the heat-shock method. From each reaction, 2 µl from the ligation were transferred to 17 x 100 polypropylene tubes and put on ice. JM109 High Efficiency Competent cells were thawed in ice, and 100 µl were added to each reaction tube. Each tube was gently flicked several times for proper mixing and placed on ice for 10 minutes. After, they were held in a 42°C water-

bath for 50 seconds, and then, sat on ice for another two minutes. In the recovery step, 900 µl of Suppression of Catabolism (SOC) media was added. Each reaction was then incubated in a 37°C shaker for 60 minutes.

### *Plating and Selection*

Each sample underwent a dilution series prior to plating. Three separate tubes were produced: a tube that contained the original concentration of the plasmid; a  $10^{-1}$  dilution; and a  $10^{-2}$  dilution. Each tube was plated on LB selection media (per liter: 10g tryptone, 5g yeast extract, 5g NaCl, 0.25 ml of 20 mg/ml X-gal, 1 ml of Ampicillin stock at 100 mg/ml, and 5 ml of IPTG stock). These plates were incubated for 24 hours at 37°C. Colonies were selected by Ampicillin resistance. Colonies containing the DNA insert were screened by lack of  $\beta$ -galactosidase cleavage indicated by a white color. Successful transformations were then re-plated onto a master plate, where they were assigned numbers.

### *Colony PCR: plasmid amplification*

Selected plasmids were then subjected to colony PCR to be amplified. The cycling conditions were:

- 1) A 95°C step for 3 minutes;
- 2) a 95°C denaturation, a 50°C annealing step, and a 68°C extension step, all of which ran for 40 cycles.
- 3) A 68°C step for 3 minutes;
- 4) an 8°C hold.

The colony PCR was conducted in 15 µl reactions, in the same ratios as the aforementioned PCR step with a few exceptions: DNA was obtained by stabbing a sterile toothpick into a colony on the master plate and dipping into the PCR mix. SP6 and T7 primers were used to utilize the promoters flanking the insertion site on the plasmid. As before, working concentrations for both were both 10 µM (respectively, the sequences are 5'-TATTTAGGTGACACTATAG-3' and 5'-TAATACGACTCACTATAGGG-3').

The colony PCR was checked by gel electrophoresis, as indicated earlier, for a DNA fragment of 1379 bp in length.

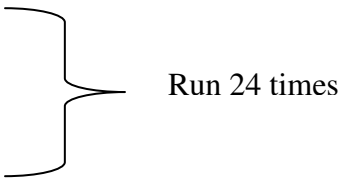
#### *Colony PCR cleanup*

The colony PCR was purified by the Exosap-it protocol (USB). 5 µl of each colony PCR product was added to 2 µl of the Exosap-it reagent. Each of the samples was incubated at 37°C for 15 minutes and again, at 80°C for 15 minutes.

#### Sequencing

##### *Sanger Sequencing Reaction*

The purified DNA was subjected to the following sequencing conditions using Big Dye sequencing master mix:

- 1) 95°C for 5 minutes;
  - 2) 95°C for 30 seconds;
  - 3) 55°C for 20 seconds;
  - 4) 60°C for 4 minutes;
  - 5) 72°C for 10 minutes;
  - 6) 4°C for infinity ie, until needed.
- 

The SP6 primer was designated the reverse primer, while the T7 primer was the forward. Each sample had two tubes, each counting one primer among its reagents. The reagents were added for a combined 5 µl as follows: 0.8 µl of primer at 1 µM; 1 µl of DNA; 0.75 µl of Big Dye; 2.45 µl sequencing grade dH<sub>2</sub>O.

#### *Sequencing precipitation & Chromatogram*

Precipitation of the Sanger reaction was as follows: 4 µl of sequencing grade dH<sub>2</sub>O and 16 µl of 95% ethanol was added to each sample in a 1.5 ml centrifuge tube – these tubes were vortexed and left to sit at room temperature for 15 minutes; they were centrifuged for 20 minutes at 4°C and 14000 rpm; next, the supernatant was removed. 125 µl of 70% ethanol was added to each sample and vortex. The tubes were spun for another 10 minutes at 4°C and 14000 rpm; the supernatant was then removed. Finally, the tubes were heated to 95°C for 5 minutes with the lids open.

The sequences were read by a 3130 Applied Biosystems sequencer by capillary electrophoresis. Each sequencing sample produced a corresponding chromatogram.

#### *Infection experiments: LD50*

##### Finding the conversion factor

After bacterial isolates Pink Egg 1&2 were sequenced and BLAST searches *Phaeobacter*, infection experiments began. *Phaeobacter* was grown to log phase in liquid SWT culture in a 28°C over 48 hours. Twenty-fold dilutions of this culture were plated four consecutive times; the original culture was also plated. The last plate reported an average of about 265 cells/ml. The concentration of cells in the initial culture was

determined to be 4.24 E7 cells/ml. This value was divided by and Absorbance at 600 nm ( $A_{600}$ ) of 1.883. Thus, the conversion factor reported was 2.25 E7 CFU/ml/ $OD_{600}$ .

#### Serial dilutions

The concentration (in Colony Forming Units/ ml of SWT) of each liquid culture of *Phaeobacter* was obtained by spectrophotometer measurement. Concentrations were accepted if the accompanying  $A_{600}$  measurement was less than 1.000 to guarantee acceptable linear relationship between CFU/ml and  $OD_{600}$ .

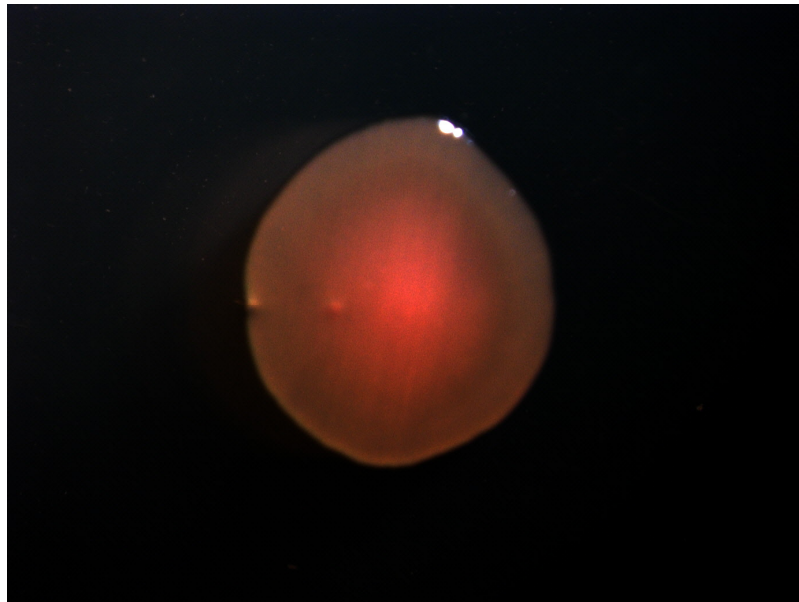
For the experiment, 10 juvenile aposymbiotic squid were infected at designated concentrations ranging from  $10^6$  to  $10^3$  CFU/ml. The concentration of the initial culture was measured by a spectrophotometer. To obtain a value of  $10^6$  cells/ml, a theoretical value of  $10^8$  cells/ml was divided by the measured concentration in cells/ 100 ml; in the first trial, the result was 1.6 ml of liquid culture. This amount was spun down, resuspended in 100  $\mu$ l of  $dH_2O$ , and added to a beaker with 100 mls of filtered seawater labeled, " $10^6$ ." This beaker underwent three ensuing ten-fold dilutions into beaker demarked,  $10^5$ ,  $10^4$ , and  $10^3$ , respectively. From each beaker, ten glass vials were filled with 5 mls of infected seawater. One set of ten vials was filled with normal filtered seawater as a negative control. One juvenile squid was placed into each of the 50 glass vials and observed over a four to five day period. If no change in the health of the animal was observed within the first two days of infection, a secondary water change was executed. This water was infected with a new *Phaeobacter* liquid culture, diluted as aforementioned.



## **Results**

### *Phaeobacter from egg clutches*

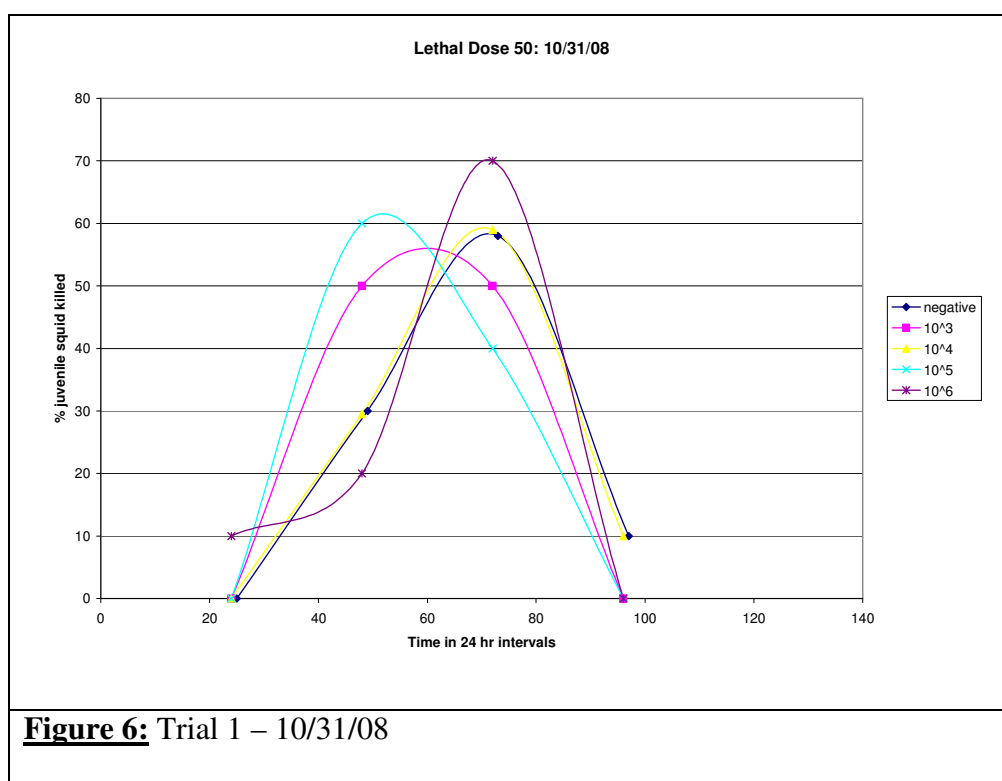
Molecular 16s rDNA sequencing analysis revealed that the microbe cultured from the juvenile egg case was *Phaeobacter*. This was supported by NCBI BLAST results returning *Phaeobacter gallaeciensis* with 97 % identity , 99% coverage of the 16s gene, and and E value of 0.0.; The next highest blast result was *Phaeobacter daeponensis* with 97% identity and an E value of 0.0. *Phaeobacter* is a genus of  $\alpha$ -proteobacterium within the Rhodobacteraceae family. Preliminary characterization of *Phaeobacter* isolate revealed a gram negative, motile, oxidase positive bacterium. A related bacterium has been sequenced from the ANG of several other marine cephalopods. In the North Atlantic squid species, *Loligo pealei*, *Roseobacter* has been identified as a dominant member of the ANG consortia<sup>5</sup>. Later studies have reclassified some species of *Roseobacter* as *Phaeobacter*<sup>6</sup>.



**Figure 5:** Colony of *Phaeobacter*

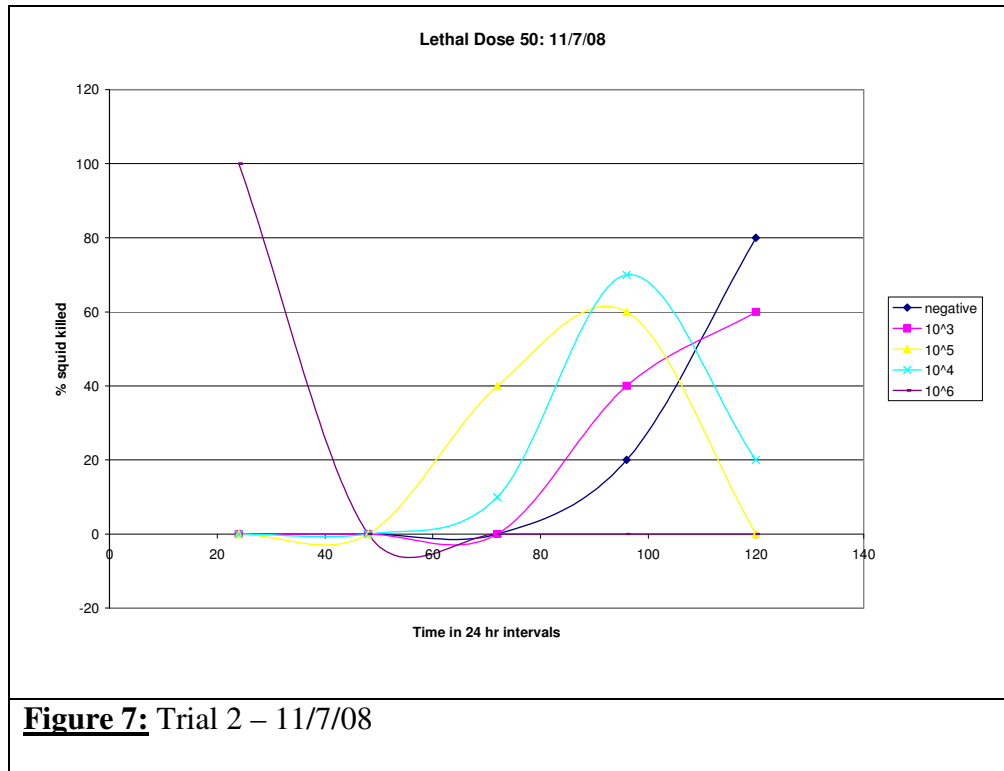
### *LD<sub>50</sub> infection experiments*

The Median Lethal Dose (LD<sub>50</sub>) is the amount of a pathogen required to induce disease in half of an exposed population. Thus, LD<sub>50</sub> trials were carried out to establish whether *Phaeobacter* had pathogenic qualities. Four trials were conducted in this study.



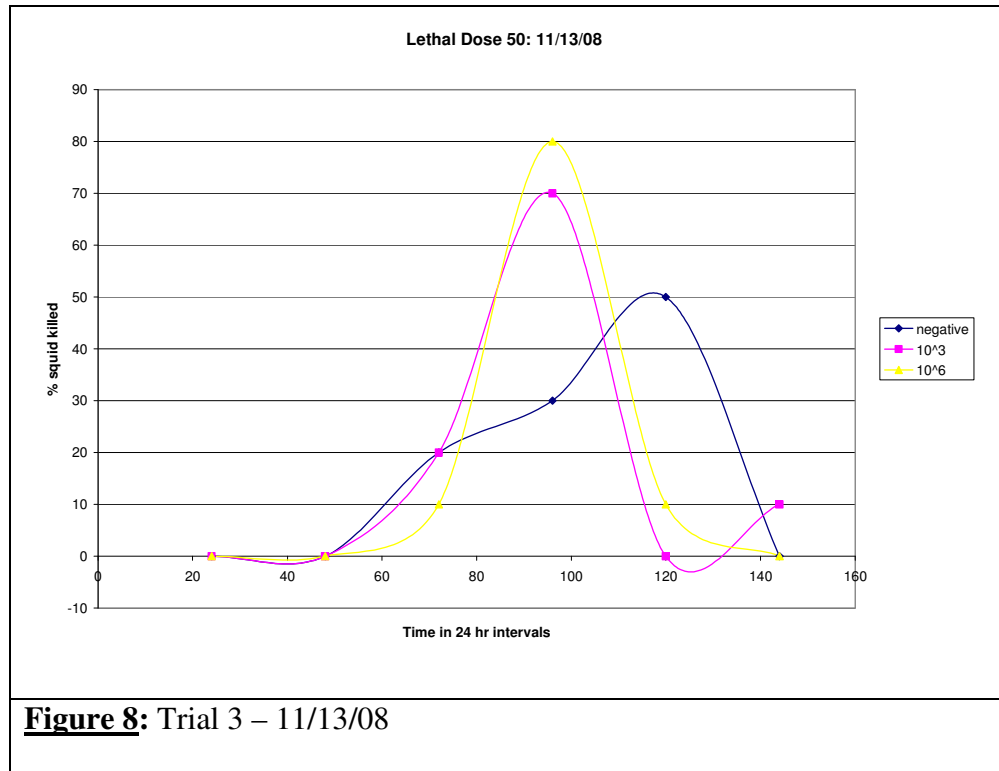
In the first LD<sub>50</sub> trial, juvenile squid displayed a slight effect to the varying dosages of *Phaeobacter*. At the first time point, ten percent of squid died in vials where *Phaeobacter* was most concentrated. By the 48 hour time point, the response was more obscure; squid from all lower concentrations displayed higher percentages of mortality than the vials containing 10<sup>6</sup> cells/ml. The third time point denotes the mortality of nearly

the entire squid population, signifying that time had become a factor in the deaths of the subjects.

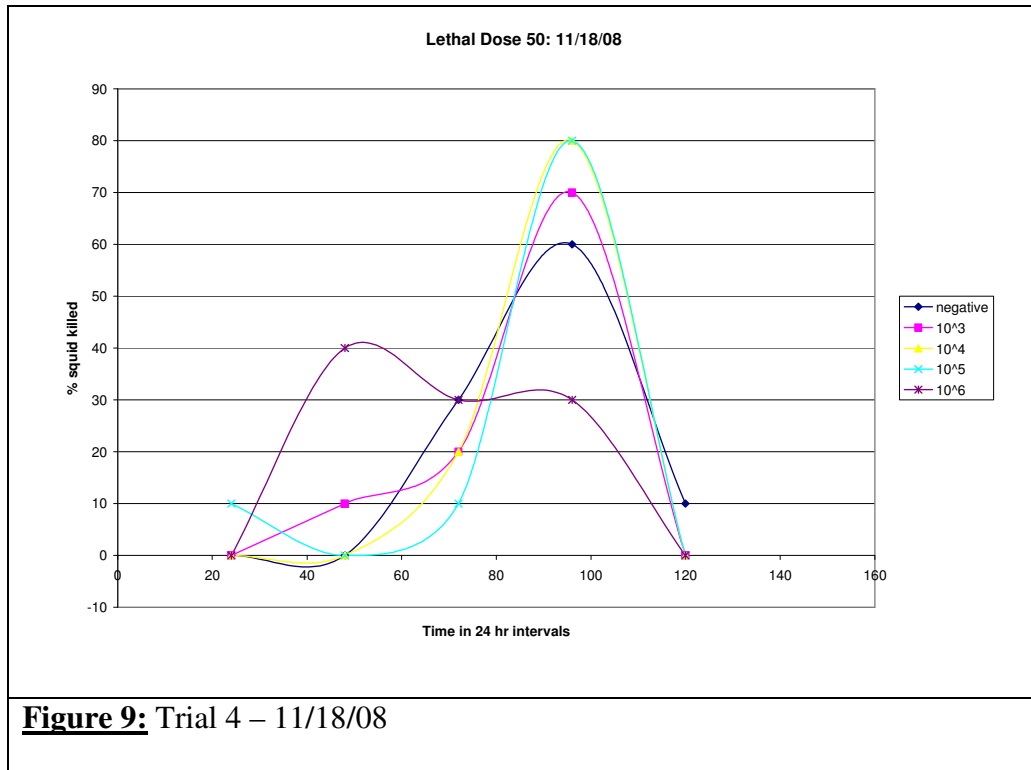


In the second LD<sub>50</sub> experiment, juvenile squid were more responsive to increasing concentrations of *Phaeobacter*. At the first time interval, the entire population of juveniles in the 10<sup>6</sup> cell/ ml vials had died. By the 72 hour mark, 40 percent of squid in the 10<sup>5</sup> cells/ml vials had died, and 10 percent from the 10<sup>4</sup> cells/ ml vials had died; the juveniles at lower concentrations were able to survive completely. At the same time, a secondary water change was made with a newly grown culture of *Phaeobacter*. At the fourth day of infection, juveniles seemed to be reaching a terminal period in the vials as most of the squid died. However, the squid in the 10<sup>5</sup> and 10<sup>4</sup> cells/ ml vials displayed greater percent mortality than did squid at lower concentrations; furthermore, twice as

many squid in the  $10^4$  vials died than in the negative control. By the final day, all remaining juveniles died.

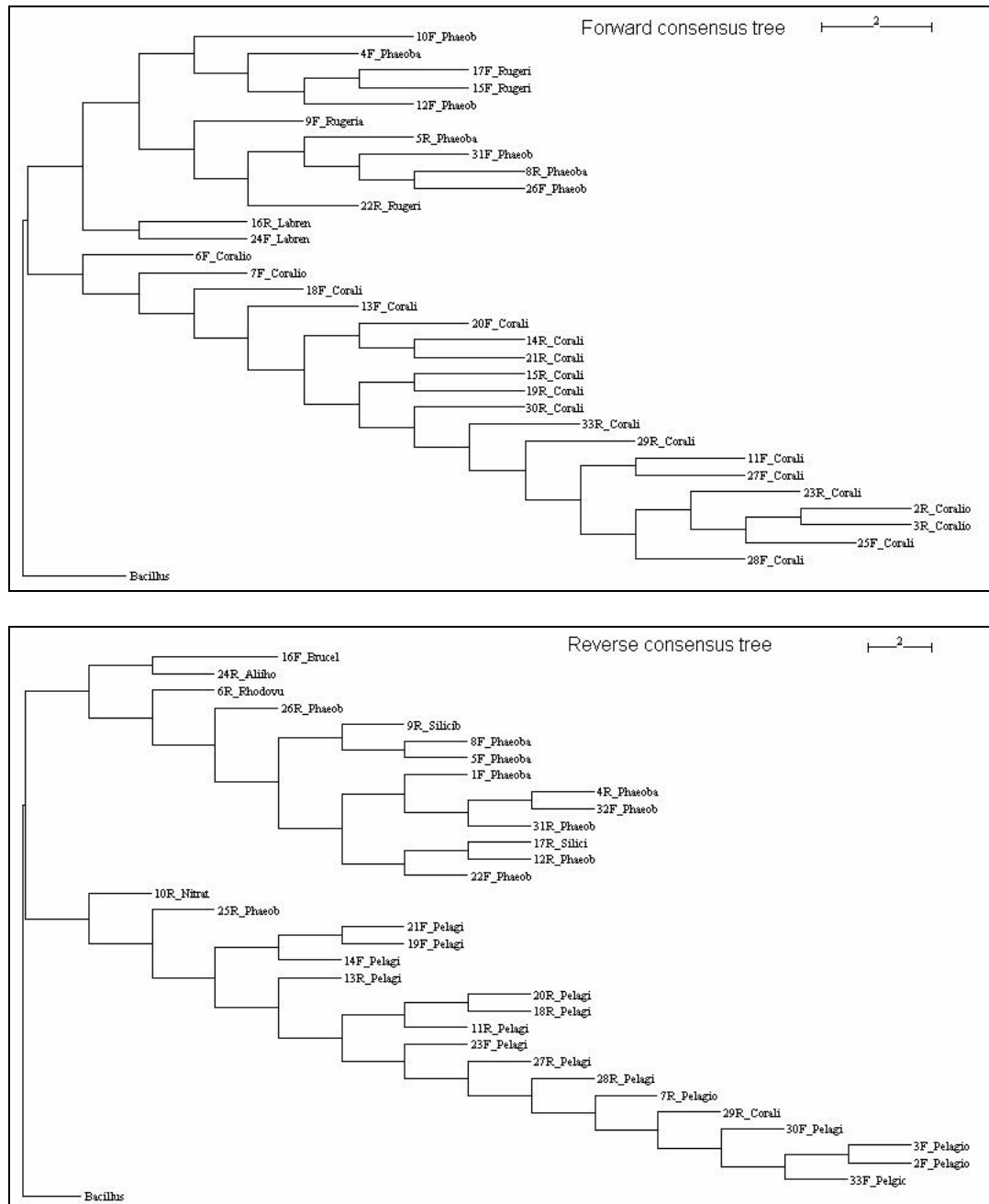


Only thirty squid were available for the third  $LD_{50}$  trial. The most significant feature in this data set occurs at 96 hours when 80 percent of the  $10^6$  constituents and 70 percent of the  $10^3$  constituents die off, and only juveniles in the negative control survive. The disparity reflects the appropriate dosage effect. Since 50 percent of juveniles in the negative control survive to the next time point, time was not a factor in juvenile mortality.



As evident at the 48 hour time point in the final trial, juveniles at the highest concentration were initially most sensitive to *Phaeobacter* in the final LD<sub>50</sub> trial. At the same stage, the juveniles of lower concentrations displayed some irregularity since the next largest response was from the  $10^3$  population. After 96 hours had passed, nearly all of the subjects died, with juveniles in the  $10^4$  and  $10^5$  vials displaying 80 percent mortality.

## 16s rDNA sequencing of the ANG



**Figure 10:** Forward and Reverse Consensus Phylogenic Trees

In the isolation of bacterial DNA from ANG tissue, 96 transformants were chosen by blue/white screening using X-gal. Colony PCR amplification and gel electrophoresis has identified 70-80 with the desired insert size. Thus far, 33 of these colonies have been sequenced and BLAST results identify potentially 10 different bacterial species.

Sequencing results from the ANG identified *Phaeobacter* as the dominant member of the 33 sequences in the library, comprising up to 30% of the forward sequences and 21% of the reverse sequences. In the forward direction, 30% of the sequenced bacteria were identified as *Coraliomargarita akajimensis* and nearly 24% were recognized as *Pelagicoccus croceus*. In the reverse direction, 30% of the sequenced bacteria were also identified as *Coraliomargarita akajimensis* and 21% were recognized as *Pelagicoccus croceus*. While the *Phaeobacter* BLASTs were recovered with 96 -99% identity, *Coraliomargarita akajimensis* and *Pelagicoccus croceus* scored relatively lower with a range of 87-89% identity. Also, the library shows a disparity between the BLAST results of complementary forward and reverse sequences. These discrepancies are due to the inherent inability of the Big Dye chain-termination reaction to generate enough truncated product to read an entire 16S gene with capillary electrophoresis.

Several bacteria were present as minority constituents in the sequencing of the ANG gene library. *Silicibacter lacuscaerulensis*, a moderately halophilic bacterium native to Iceland, was identified in a reverse sequence with 97% identity. *Rhodovulum kholense* is a mud bacterium native to Khola, India that was identified in Reverse sequence with 95% identity. Likewise, *Labrenzia aggregate*, an inhabitant of sediment in the Baltic sea, was identified in a forward sequence with 93% identity. *Aliihoeflea*

*aestuarii* is a novel member of the *Phyllobacteriaceae* family that was found as a reverse sequence with 94% identity. *Ruegeria* is a marine agrobacterium that was found in forward and reverse sequences, all with 97% identity. Finally, *Nitratireductor basaltis* is a denitrifying bacterium native to Korea that was identified in a reverse sequence with 90% identity.

The BLAST results from the ANG library were used to construct two consensus phylogenetic trees by combining trees made with maximum parsimony and maximum likelihood methods. One consensus tree contained all the forwards sequences, while the other contained the complementary reverse sequences. Overall, both trees exhibit similar structure. Both feature two major clades, and the chosen gram positive out-group, *Bacillus cereus*. Each clade is dominated by a major species, and also feature relatively similar sequences, which BLAST identify as other species.

When comparing the two trees, dissimilarities become evident. Clade B highlights the major difference between the forward and reverse sequences. When blasted, the forward sequences of Clade B are dominated by *Coralimargarita akajimensis*. The corresponding reverse sequences are dominated by *Pelagiococcus croceus*. This incongruity can also be seen on a smaller scale in isolated parts of both trees.

In both trees, Clade A was dominated by *Phaeobacter*. In the forward consensus tree, the first major division in this clade was comprised of organisms from the novel genus, *Labrenzia*. Further, *Ruegeria* was present in 3<sup>rd</sup>, 4<sup>th</sup>, and 5<sup>th</sup> subdivisions. In the reverse consensus tree, the first major division showed sequence similarity between a microorganism from the genus, *Brucella*, and *Aliihoeflea aestuarii*. A second division in the neighboring branch highlighted a distinction made by *Rhodovulum kholense*. Later



subdivisions demonstrate some sequence similarity between the featured members of the *Phaeobacter* and *Silicibacter* genera. Other than *Ruegeria*, It is important to note that some outliers like *Nitratireductor* were unique to their respective trees.

## *Discussion*

Colonization of the small intestine by *Vibrio cholerae* is responsible for the gastrointestinal affliction, cholera. However, the same bacterium lives commensally with a variety of marine life including crustaceans and algae. The behavior of this microbe conveys a natural paradox – that under certain circumstances, benign associations can become pathogenic. With this premise, it became logical to suspect the pink egg bacterium, *Phaeobacter*, as a potential causative agent in the deaths of the juvenile egg cases, and as a normal microbial resident of Hawaiian bobtail squid. This inquiry led to the first known microbial characterization of the ANG in *E. scolopes* by 16s sequencing analysis.

The confirmation of *Phaeobacter* in the ANG consortia presents a probable transition to the squid egg cases because of the suspected function of the gland in related species of squid. *Phaeobacter* constituted a dominant portion of the potentially different bacteria isolated in the ANG sequence library; if these results reflect normal trends in the gland, it is possible that *Phaeobacter* could be transmitted preferentially to the gelatinous egg cases. It is highly unlikely that contamination could have compromised results since *Phaeobacter* is a genus that has been previously localized to isolated regions of the world such as Korea's Yellow Sea. Furthermore, laboratory parameters work to minimize contaminants – for example, the seawater used to house the squid was synthetic and run through a 0.2  $\mu$  filter in order to eliminate microbial variables. Sequencing results sufficed in drawing preliminary conclusions about the source of the *Phaeobacter* isolated on the egg cases of *E. scolopes* – these inferences will need to be confirmed by more

definitive methods. One possible direction may be using GFP (Green Fluorescent Protein) to visualize the avenue *Phaeobacter* takes from the ANG to the squid egg cases.

The search for the source of *Phaeobacter* resulted in the discovery of 10 potentially different genera of bacteria represented in the ANG. These bacteria are evident in the phylogenic consensus trees of Figures E & F. The discrepancy in clade B, between forward and reverse consensus trees, indicates that each sequence may either be *Pelagiococcus croceus* or *Coralimargarita akajimensis*; thus, clade B has the potential to feature up to two genera of bacteria. Clade A, in both the forward and reverse consensus trees, displays an overall bacterial lineage that is consistent with the *Roseobacter* clade, which includes 40 genera of bacteria (Roseo2008). A potential trend may be emerging as the sequenced isolates, *Pheobacter gallaeciensis*, *Silicibacter lacuscaerulensis*, and a species from the genus *Ruegeria*, are all members of this clade. These three species were also recovered from the ANGs of squids belonging to the *Loliginidae* family <sup>5</sup>. It will be interesting to see whether further sequencing yields any more related bacterial species since the *Roseobacter* clade features an impressive diversity of microbiota – some are capable of producing bacteriochlorophyll *a* and perform aerobic anoxygenic photosynthesis <sup>7</sup>. In comparison to the ANG sequencing project, results from the *Phaeobacter* infection experiments were less conclusive.

There is a dearth of information about *Phaeobacter* in circulating literature. A recent study has reclassified *Roseobacter gallaeciensis* to *Phaeobacter gallaeciensis* <sup>6</sup>. Other studies have localized *Phaeobacter daeponensis* to tidal flats in the Yellow Sea <sup>8</sup>. It is also known that one species of *Phaeobacter* behaves as a probiotic agent to the fish

species, the Danish turbot <sup>9</sup>. However, a species of bacteria within the same clade, *Roseovarius crassostreae*, has been reported as the pathogenic agent responsible for Juvenile Oyster Disease in several species of oysters <sup>10</sup>. These publications illustrate that the species from the *Phaeobacter* genus are capable of succeeding in a variety of environmental niches. While it is privy to benign and sometimes beneficial associations with other organisms, there is a potential for pathogenesis simply by virtue of being a bacterium. As evidenced by *V. cholerae*, a microbe may exhibit pathogenic behavior when in different environmental niches. Alternatively, virulence is transferable between bacteria by horizontal gene transfer, which can consequently affect entire bacterial populations <sup>11</sup>. Although it may be difficult to assign a cause virulent behavior, it is evident that the transition can be accomplished with relative facility, since pathogenic and symbiotic interactions utilize mutual genetic and regulatory mechanisms.

The collected data from the LD<sub>50</sub> infections did not clearly resolve the role of *Phaeobacter* in the mortality of *E. scolopes* juveniles. Nonetheless, the experiments presented a few prospective trends in the interaction of *Phaeobacter* and the host squid. It can be perceived that at higher concentrations of *Phaeobacter*, juvenile squids are more inclined to die early when compared to the negative control (Figures B and D). In three of the experiments, juveniles from the 10<sup>6</sup> concentrations of *Phaeobacter* were the first group to exhibit a significant percent mortality. A clear dosage effect is also visible in Figure C as more juveniles died at the 10<sup>6</sup> concentration than at the 10<sup>3</sup> concentration; however, these results are undermined by graphical irregularities such as those observed in Figure A. When taken together, the data gathered from the LD<sub>50</sub> infections are insufficient to clearly establish whether *Phaeobacter* is pathogenic to juvenile squid.

Conducting more of these infection experiments will differentiate inconsistent results from those deserving merit. An alternative approach will subject juvenile eggs to *Phaeobacter* infection, mimicking the original conditions of the disease. It is possible that the innate immune system of the juvenile squid provides a degree of tolerance to pathogenesis that is absent in egg clutches – that squid eggs are inherently more vulnerable to pathogenesis.

## **Literature Cited**

1. Nyholm, S. V., and M. J. McFall-Ngai. "The Winnowing: Establishing the Squid-*Vibrio* Symbiosis." Natural Reviews Microbiology 2 (2004): 632–642.
2. Ruby, Edward G. "Lessons from a Cooperative, Bacterial-Animal Association: The *Vibrio fischeri* – *Euprymna scolopes* Light Organ Symbiosis." 1996. NCBI. Homer Babbidge Library, Storrs. 2009 <<http://ncbi.nlm.nih.gov/>>.
3. Nyholm, S. V. "Recognition between Symbiotic *Vibrio fischeri* and the Haemocytes of *Euprymna scolopes*."
4. Biggs, James, and David Epel. "Egg Capsule Sheath of *Loligo opalescens* Berry; Structure and Association with Bacteria." The Journal of Experimental Zoology (1991): 263-67.
5. Pichon, Delphine, Valeria Gaia, Mark D. Norman, and Renata Boucher-Rodoni. "Phylogenetic Diversity of Epibiotic Bacteria in the Accessory Nidamental Glands of Squids (Cephalopoda: Loliginidae and Idiosepiidae)." Marine Biology (2005): 1323-332.
6. Martens, Torbern, Thorsten Heidorn, Rudiger Pukall, Meinhard Simon, Brian J. Tindall, and Thorsten Brinkoff. "Reclassification of *Roseobacter gallaeciensis* Ruiz-Ponte et al. 1998 as *Phaeobacter gallaeciensis* gen. nov., comb. nov., description of *Phaeobacter inhibens* sp. nov., reclassification of *Ruegeria algicola* (Lafay et al. 1995) Uchino et al. 1999 as *Marinovum algicola* gen. nov., comb. nov., and emended descriptions of the genera *Roseobacter*, *Ruegeria* and *Leisingera*."

7. Brinkhoff, Thorsten, Helge-Ansgar Giebel, and Meinhard Simon. "Diversity, Ecology, and Genomics of the *Roseobacter* clade: a Short Overview." 6 Feb. 2008. NCBI. Homer Babbidge Library, Storrs. April & May 2009 <<http://ncbi.nlm.nih.gov/>>.
8. Yoon, Jung-Hoon, So-Jung Kang, Soo-Young Lee, and Tae-Kwang Oh. "*Phaeobacter daeponensis* sp. nov., Isolated from a Tidal Flat of the Yellow Sea in Korea." International Journal of Systemic and Evolutionary Microbiology 57 (2008): 856-61.
9. Porsby, Cisse H., Kristian F. Nielsen, and Lone Gram. "*Phaeobacter* and *Ruegeria* Species of the *Roseobacter* Clade Colonize Separate Niches in a Danish Turbot (*Scophthalmus maximus*)-Rearing Farm and Antagonize *Vibrio anguillarum* under Different Growth Conditions." Applied and Environmental Microbiology 74 (2008): 7356-364.
10. Boardman, Cynthia L., Aaron P. Maloy, and Katherine J. Boettcher. "Localization of the Bacterial Agent of Juvenile Oyster Disease (*Roseovarius crassostreae*) within Affected Eastern Oysters (*Crassostrea virginica*)." Sept. 2007. NCBI. Homer Babbidge Library, Storrs. April & May 2009 <<http://ncbi.nlm.nih.gov/>>.
11. Hentschel, Ute, Michael Steinert, and Jorg Hacker. "Common Molecular Mechanisms of Symbiosis and Pathogenesis." Trends in Microbiology 8 (2000): 226-30.

12. Hanlon, Roger T., Michael F. Claes, Susan E. Ashcraft, and Paul V. Dunlap.

"Laboratory Culture of the Sepiolid Squid *Euprymna scolopes*: a Model System for Bacteria-Animal Symbiosis." The Biological Bulletin (1997): 364-74.